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Article

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Achieving Stable Mainstream Nitrogen Removal via the Nitrite Pathway by Sludge Treatment Using Free Ammonia

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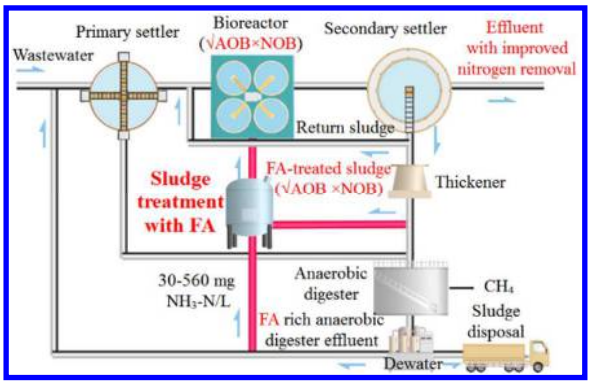
TOC Art


Primary settler
Wastewater
Bioreactor
(√AOB×NOB)
Secondary settler
Effluent
with improved
nitrogen removal
Return sludge
Sludge
treatment
with FA
FA-treated sludge
(√AOB ×NOB)
Thickener
30-560 mg
NH3-N/L
Anaerobic
digester
CH4
Sludge
disposal
FA rich anaerobic
digester effluent
Dewater

ABSTRACT

Biological nitrogen removal through the nitrite pathway ($NH_4^+ \rightarrow NO_2^- \rightarrow N_2$) is favorable for the wastewater treatment plants without sufficient carbon source. This study demonstrates an innovative approach for attaining the nitrite pathway based on sludge treatment using free ammonia (FA i.e. NH_3). This approach is based on our innovative discovery in this study that FA at 210 mg NH_3-N/L is far less biocidal to ammonium oxidizing bacteria (AOB) than to nitrite oxidizing bacteria (NOB). Twenty-two percent of the activated sludge from the sequencing batch reactor (SBR) receiving synthetic domestic wastewater was treated in an FA treatment unit at 210 mg NH_3-N/L for one day. The FA treated sludge was afterwards recirculated back to the SBR. A nitrite accumulation ratio of above 90% was quickly achieved (in 40 d) and maintained stable in the SBR, indicating the establishment of the nitrite pathway. The NOB population and activity after implementing FA treatment was less than 5% of those without FA treatment, suggesting the washout of NOB. In contrast, the AOB population and activity in the SBR were not affected. The nitrogen removal performance was significantly improved after incorporating the FA approach. The FA approach is a closed-loop approach and is economically and environmentally attractive.

INTRODUCTION

The conventional biological nitrogen removal process in wastewater treatment systems is achieved by complete oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) (nitrification, i.e. $NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) followed by the reduction of nitrate (NO_3^-) to nitrogen gas (N_2) (denitrification, i.e. $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Both nitrification and denitrification involve nitrite (NO_2^-) as an intermediate. If nitrification is terminated at nitrite ($NH_4^+ \rightarrow NO_2^-$, i.e. nitritation), denitritation from nitrite to nitrogen gas ($NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) can be attained (i.e. nitrogen removal via the nitrite pathway). Nitrogen removal via nitrite instead of nitrate can decrease oxygen demand by 25%, reduce chemical oxygen demand (COD) requirement by 40% and also reduce sludge production accordingly.[1] Nowadays, many wastewater treatment plants (WWTPs) face the problem of insufficient COD for biological nitrogen removal via nitrate, where external carbon sources (e.g. methanol, ethanol) have to be added to meet the regulatory nitrogen discharge standard and thus the operating cost of the WWTPs will increase. Therefore, the reduction in COD requirement via the achievement of the nitrite pathway will be significantly beneficial for the WWTPs deficient in COD. The key point of attaining the nitrogen removal through nitrite pathway is to selectively wash out nitrite oxidizing bacteria (NOB; $NO_2^- \rightarrow NO_3^-$) from the system while retaining ammonium oxidizing bacteria (AOB; $NH_4^+ \rightarrow NO_2^-$).

Lots of approaches have been proposed to eliminate NOB, such as short sludge retention time (SRT) in combination with high temperature,[2] low pH,[3] aeration duration control[4,5] and low dissolved oxygen (DO).[6,7] Nevertheless, these approaches either request special growth

conditions (e.g. low pH and high temperature) or can only be applied to the intermittent aeration systems (e.g. aeration duration control), or adversely decrease AOB activity (e.g. low DO).[8] Therefore, these approaches have drawbacks and/or are unsuitable for eliminating NOB in the mainstream wastewater treatment systems and alternative approach is required.

Free nitrous acid (FNA i.e. HNO_2) has been shown to have a stronger inhibitory effect on NOB than on AOB.[9-11] For example, 0.0175 mg HNO_2-N/L (i.e. 76 mg NO_2^--N/L at pH 7.0) was found to inhibit NOB by 50%,[10] whereas up to 0.2 mg HNO_2-N/L (i.e. 850 mg NO_2^--N/L at pH 7.0) was required to inhibit AOB by 50%.[11] The inhibition of FNA on NOB has been successfully used to wash out NOB in the side stream reactor treating anaerobic digester effluent,[11,12] which has 1.0~2.0 g NH_4^+-N/L. However, it is impossible to eliminate NOB in the mainstream bioreactor treating domestic wastewater based on the inhibition of FNA on NOB. This is because the ammonium concentration in domestic wastewater is normally less than 60 mg NH_4^+-N/L and therefore the cumulative nitrite concentration in the mainstream bioreactor would seldom reach the NOB inhibition threshold. Recently, Wang et al. [13] found that FNA has a stronger biocidal effect on NOB than on AOB at a concentration that is much higher than the previously reported inhibitory concentration. Based on this finding, 80% of NOB was successfully eliminated and a nitrite accumulation ratio (NO_2^--N/(NO_2^--N+NO_3^--N)×100%) of above 80% was achieved in the mainstream bioreactor.[13] This was achieved incorporating an FNA treatment unit (1.35 mg HNO_2-N/L for 24 h) in the sludge return line to treat 22% of the sludge from the mainstream bioreactor every day. For this approach, FNA comes from the in-situ production via side stream nitritation of anaerobic digester effluent to avoid the input of addition nitrogen load.[13] Unfortunately, the side stream nitritation reactor does not exist in most of the current WWTPs.

Consequently, those WWTPs have to build the side stream nitritation reactor so as to employ the FNA approach, requiring significant capital investment.

Free ammonia (FA i.e. NH_3) also has a stronger inhibitory effect on NOB than on AOB.[14-16] For example, Vadivelu et al. [15] demonstrated that the FA inhibition on NOB initiated at below 1 mg NH_3-N/L, whereas no inhibition on AOB was observed when the FA concentration was 16 mg NH_3-N/L.[14] Analogous to FNA, we hypothesize that NOB are also likely to be eliminated from the mainstream bioreactor using sludge treatment by FA. This can be achieved incorporating an FA treatment unit in the sludge recycling line to treat part of the sludge from the mainstream bioreactor daily at a much higher FA concentration than the previously reported inhibition concentration. Additionally, the FA approach does not depend on the side stream nitritation reactor because FA is attainable directly from anaerobic digester effluent, which has 1.0~2.0 g NH_4^+-N/L at a pH of 7.5~8.6 and 33 ˚C (equal to 30-560 mg NH_3-N/L).[17,18]

The aim of this study is to assess whether it is feasible to eliminate NOB and achieve nitrogen removal through nitrite pathway via sludge treatment using FA. One lab-scale sequencing batch reactor (SBR) receiving synthetic domestic wastewater was operated, which consisted of a Baseline phase and an Experimental phase. The Baseline phase served as a control and the Experimental phase included an FA treatment unit to treat the sludge. The inactivation effect of FA treatment on the AOB and NOB activities at a selected FA concentration (210 mg NH_3-N/L) was assessed first by batch tests. Afterwards, the nitrite accumulation ratio, populations and activities of AOB and NOB, together with the nitrogen removal performance in both the

Baseline and Experimental phases of the SBR were evaluated and compared. Economic and environmental potential of the FA approach were also assessed.

MATERIALS AND METHODS

Reactor Operation and Monitoring

A lab-scale SBR with a working volume of 8 L was applied to conduct this study, seeded with the sludge from a local domestic WWTP (Brisbane, Australia). The SBR was operated in two phases (i.e. Baseline phase and Experimental phase) for more than 200 days. The Baseline phase served as a control and the Experimental phase included an FA treatment unit for sludge treatment (Figure 1).

(Position for Figure 1)

- Baseline phase (Day -90-0): The SBR was operated at 6 h per cycle comprising anoxic feeding (10 min), anoxic reaction (100 min), aerobic reaction (180 min), sludge wasting (5 min), settling (60 min) and decanting (5 min) phases. In the feeding phase of each cycle, 2 L of synthetic wastewater and synthetic FA containing NH_4HCO_3 solution mixture was added to the SBR, leading to a hydraulic retention time (HRT) of 24 h. The sludge retention time (SRT) was kept at 15 d by wasting 533 ml of mixed liquor each day. A magnetic stirrer at 250 rpm was used to mix the reactor in the feeding, anoxic, aerobic and wasting periods. Dissolved oxygen (DO) in the reactor was continuously monitored using a DO meter (miniCHEM) and maintained at around 1.5 mg/L by a programmed logic controller (PLC). pH was not controlled but measured with a pH meter

(miniCHEM), varying between 7.0 and 7.3 over a cycle. The SBR was operated at 22 ± 2°C for around 90 d during the Baseline phase to attain steady state (i.e. Steady State I), which is reflected by the stable sludge concentration and also by the similar NO_3^--N, NO_2^--N and NH_4^+-N profiles during cycles.

- Experimental phase (Day 0-125): The operational conditions of the SBR during the Experimental phase were similar to those in the Baseline phase, with the following differences. During the Experimental phase, approximately 2280 ml of sludge from the SBR was taken out every day (~570 ml of sludge was taken out in each cycle) and then thickened to 130 ml first (from 2280 ml). After that, the thickened sludge was added into an FA treatment unit, and is subject to FA treatment at 210 mg NH_3-N/L (NH_4^+-N+NH_3-N=800 mg N/L; pH=8.9; T=22 °C) for 1 day. The FA concentration was chosen based on the findings of our previous study that 210 mg NH_3-N/L is biocidal to bacteria[19] and also based on the results of Section "Batch Tests to Evaluate the Effect of FA Treatment on the NOB and AOB Activities". It should be highlighted that this was only a proof-of-concept study and therefore only one set of FA treatment condition was tested here. During the 1 day FA treatment, NH_4^+-N concentration was almost unchanged and no additional NH_4^+-N or NH_3-N was added but sodium hydroxide (1.0 mol/L) was added automatically to maintain pH at 8.9 via PLC control. The sludge was treated at a frequency of 0.29 per reactor volume every day (i.e. (2280 ml/d)/(8000 ml)). The FA concentration was determined using the formula of $S_{(NH3\text{-}N+NH4^+\text{-}N)}\times 10^{pH}/(K_b/K_w+10^{pH})$, where $S_{(NH3\text{-}N+NH4^+\text{-}N)}$ represents the NH_3-N+NH_4^+-N concentration, K_b represents the ionization constant of the ammonia equilibrium equation, and K_w represents the ionization constant of water.[16] The K_b/K_w value was calculated via the formula of

$K_b/K_w=e^{6,344/(273+T)}$.[16] To maintain the same SRT (i.e. 15 d) as that in the Baseline phase of the SBR, 30 ml of FA treated thickened sludge, equal to ~530 ml (i.e. 2280 ml/130 ml×30 ml) of mixed liquor in the SBR, was thrown away after the treatment. Afterwards, the remainder (100 ml) of the FA-treated thickened sludge was returned to the SBR equally in every feeding period (25 ml each cycle) of the SBR. The FA treatment unit was mixed by a magnetic stirrer at 250 rpm. The SBR was operated until reaching another steady state (Steady State II).

The nitrate, nitrite and ammonium concentrations in the effluent of the SBR were measured 2-3 times a week. Cycle studies in the SBR were performed each week by analyzing the nitrate, nitrite and ammonium concentrations every 30~50 min in a cycle. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations together with sludge volume index (SVI) were determined twice a week. The NOB and AOB populations and activities at Steady States I and II were examined by fluorescence in-situ hybridization (FISH) and biomass-specific nitrate production and ammonium consumption rates, respectively.

Wastewater Composition

The synthetic wastewater was prepared using different types of components, resulting in the total Kjeldahl nitrogen (TKN) and chemical oxygen demand (COD) concentrations being 50 mg/L and 300 mg/L, respectively. The synthetic wastewater comprised per litre: 153 mg NH_4Cl (40 mg NH_4^+-N), 61 mg starch, 83 mg milk powder, 60 mg sucrose, 45 mg CH_3COONa, 29 mg yeast extract, 12 mg peptone, 13 mg K_2HPO_4, 14 mg KH_2PO_4, 600 mg $NaHCO_3$, 2.5 mg $FeSO_4 \cdot 7H_2O$, 0.44 mg $CaCl_2$, 0.19 mg $NaMoO_4 \cdot 2H_2O$, 0.19 mg $MgCl_2$, 0.13 mg $CoCl_2 \cdot 6H_2O$, 0.06 mg $ZnCl_2$,

0.06 mg $MnCl_2 \cdot 4H_2O$, 0.06 mg $CuSO_4$, 0.06 mg H_3BO_3 and 0.04 mg $NiCl_2 \cdot 6H_2O$. The synthetic FA containing NH_4HCO_3 solution was to simulate FA in the anaerobic digester effluent, resulting in an extra 20% nitrogen load to the synthetic wastewater.

Batch Tests to Evaluate the Effect of FA Treatment on the NOB and AOB Activities

The impact of FA treatment on the NOB and AOB activities was evaluated by batch tests, thereby determining whether it is suitable to apply the selected FA concentration (i.e. 210 mg NH_3-N/L) to the FA treatment unit for sludge treatment.

200 ml of mixed liquor (MLSS=1050 mg/L) was taken out from the SBR towards the end of an aerobic reaction phase and then added into a batch reactor. Following that, the NOB and AOB activities were measured (i.e. prior to FA treatment) using the method detailed in the paragraph below. Afterwards, an ammonium chloride stock solution (40 g NH_4^+-N/L) was added into the batch reactor to attain the desirable ammonium+ammonia concentration of 800 mg N/L. The pH was maintained at 8.9 ± 0.1 and the temperature was 22 ± 2°C. As such, the FA concentration was determined to be 210 mg NH_3-N/L.[16] The treatment time was 1 day. After 1 day FA treatment, the NOB and AOB activities were measured after removing FA through sludge washing using the method detailed below. The NOB and AOB activities after FA treatment were expressed as the percentages of their activities prior to FA treatment.

In order to measure the NOB and AOB activities after FA treatment, the ammonium free effluent from the SBR was first used to wash the mixed liquor to get rid of ammonium and no NH_4^+- or NH_3 was detected after washing. After that, the sodium nitrite and ammonium chloride stock

solutions (both at 4.0 g N/L) were added into the batch reactor to achieve an initial nitrite and ammonium concentration of 20 mg NO_2^--N/L and 25 mg NH_4^+-N/L, respectively. DO was maintained at above 4.0 mg O_2/L. pH was maintained at 7.0~7.3, which was the pH in the SBR. Mixed liquor samples were taken every 30 min for the analyses of nitrate and ammonium. The MLVSS concentration was determined when the activity test was finished (3 h for each activity test). The activities of NOB and AOB were determined as the biomass-specific nitrate production and ammonium consumption rates. The NOB and AOB activities prior to FA treatment were measured in a similar manner, except that no sludge washing was conducted.

Analytical Procedures

The analyses of MLSS, MLVSS, SVI, COD and TKN were performed in accordance with standard methods.[20] For the analysis of nitrate, nitrite and ammonium concentrations, the samples were filtered via disposable Millipore filter units (pore size: 0.22 μm) straight away following sampling. The concentrations of nitrate, nitrite and ammonium were measured by a Lachat QuikChem8000 Flow Injection Analyzer (Lachat Instrument, Wisconsin).

FISH was carried out to determine the percentages of AOB and NOB, as described in Daims et al. [21]. The following oligonucleotide probes were used:[22] FITC labelled EUB-mix (comprising EUB338, EUB338-II and EUB338-III) for most bacteria, Cy3 labelled Ntspa662 for *Nitrospira* genera, Cy3 labelled Nit3 for *Nitrobacter* sp., and Cy5 labelled Nso1225 for Betaproteobacterial AOB. For quantitative FISH analysis, 20 random microscopic fields were taken for each sample using a confocal laser scanning microscope (Zeiss LSM 510 Meta, Germany). The biovolume fraction of the bacteria of interest was determined via image analysis using DAIME version 1.3.

249

250 The significance of the results was assessed by an analysis of variance. $p > 0.05$ was regarded
251 statistically insignificant, whereas $p < 0.05$ was regarded statistically significant.

252

253 **RESULTS AND DISCUSSION**

254 **Inactivation of NOB and AOB by FA**

255 The percentage of AOB activity after 1 day FA treatment (210 mg NH_3-N/L) were $50 \pm 5\%$ of
256 their activity before FA treatment, whereas the NOB activity was not detected following 1 day
257 FA treatment. This clearly indicated that FA inactivated NOB more significantly than it did on
258 AOB. It should be noted that the decreased activity could not be attributed to the inhibition (i.e.
259 relying on constant presence of FA) as demonstrated in the previous studies.[14,15] This is because
260 FA was already removed via sludge washing prior to the activity tests. Therefore, the decreased
261 activity was probably because of inactivation (i.e. still no activity after FA removal).

262

263 **Biological Nitrogen Removal through the Nitrite Pathway**

264 A long-term experiment was conducted to assess whether it is effective in attaining the nitrogen
265 removal through nitrite pathway via sludge treatment using FA. To this end, the nitrite
266 accumulation ratio, NOB and AOB activities and populations, and nitrogen removal performance
267 in the SBR both before and after implementing FA treatment were assessed and compared.

268

269 Figure 2 shows the nitrite accumulation ratio in the SBR before and after including the FA
270 treatment. No nitrite accumulation was observed before incorporating the FA treatment (i.e.
271 Baseline phase). After including the FA treatment (i.e. Experimental phase), the nitrite

accumulation ratio in the SBR increased quickly during the first 40 days and then stabilized at around 90% over the remaining period of the Experimental Phase (Table 1 and Figure 2). This indicates that the nitrogen removal through nitrite pathway could be effectively established by sludge treatment using FA.

(Position for Figure 2)

The establishment of the nitrogen removal through nitrite pathway was confirmed by the NOB and AOB activities and populations. For instance, Table 1 reveals that the NOB activity at Steady State II with FA treatment (0.2 ± 0.1 mg NO_3^--N/g MLVSS/h) was only about 2% of that at Steady State I without FA treatment (8.1 ± 0.7 mg NO_3^--N/g MLVSS/h). Microbial composition analysis through FISH shows that the NOB population at Steady State II with FA treatment was around 5% of that at Steady State I without FA treatment (see Table 1). The low NOB activity and population conclusively reveal that the majority of the NOB were eliminated from the SBR after implementing FA treatment. By contrast, the AOB activity at Steady State II with FA treatment (8.7 ± 0.7 mg NH_4^+-N/g MLVSS/h) was comparable ($p>0.05$) with that (9.6 ± 0.6 mg NH_4^+-N/g MLVSS/h) at Steady State I without FA treatment. Similarly, the AOB population at Steady State II ($7.6 \pm 1.3\%$) with FA treatment was similar ($p>0.05$) to of that ($6.9 \pm 1.6\%$) at Steady State I without FA treatment (see Table 1). These indicate that the AOB activity and population were not negatively affected.

(Position for Table 1)

The effluent nitrogenous compound concentration (NH_4^+-N and NO_x^--N) and periodic cycle studies were used to assess the impact of the nitrogen removal through nitrite pathway on the biological nitrogen removal performance. Figure 3A shows the concentrations of NH_4^+-N, NO_2^--N, NO_3^--N and NO_x^--N (i.e. NO_2^--N+NO_3^--N) in a cycle of the SBR before including FA treatment. Ammonium was completely oxidized to nitrate during the aerobic phase. Nevertheless, denitrification was not fully completed during the anoxic phase, as shown by around 2.0 mg NO_x^--N/L towards the end of the anoxic phase and by an effluent NO_x^--N concentration of up to 16.4 mg N/L (Figures 3A and 4, and Table 1). This suggests that the organic carbon in the wastewater was inadequate for full denitrification. In contrast, no NO_x^--N was observed at the end of the anoxic phase after implementing FA treatment (Figure 3B). This was accompanied by a lower effluent NO_X^--N concentration compared with the phase without FA treatment (11.5 ± 0.1 vs. 16.4 ± 0.5 mg N/L) (Table 1 and Figure 4), despite the nitrogen load before and after implementing FA treatment was identical in the SBR. This clearly demonstrates the advantage of the nitrogen removal through nitrite pathway in improving the biological nitrogen removal performance while the wastewater does not contain adequate carbon source. The enhanced nitrogen removal performance could be attributed to the decreased carbon request for biological nitrogen removal in the presence of the nitrite pathway.[1]

(Position for Figure 3)

(Position for Figure 4)

The MLSS concentrations in the SBR before and after FA treatment were determined to examine the impact of implementing FA treatment on sludge concentration. Table 1 shows that the sludge concentration at Steady State I without FA treatment (1048 ± 57 mg/L) was higher ($p<0.05$) than that (850 ± 54) at Steady State II with FA treatment. This resulted in a reduced sludge concentration by 18%. This was likely due to the fact that the sludge biodegradability was enhanced after FA treatment as previously observed in the other study.[19]

The SVI at Steady State I without FA treatment (193 ± 25 ml/g) was comparable ($p=0.17$) with that (244 ± 24 ml/g) at Steady State II with FA treatment (Table 1), indicating that sludge settleability was not significantly affected by FA treatment.

Closed Loop Concept of FA Approach in Wastewater Treatment

This study reveals for the first time that sludge treatment using FA is effective in washing out NOB and establishing the nitrogen removal through nitrite pathway in the mainstream wastewater treatment systems. This was experimentally demonstrated through a long-term experiment in one sequencing batch reactor. A nitrite accumulation ratio of above 90% was stably achieved after implementing FA treatment, which led to improved nitrogen removal performance without adding any extra carbon sources.

Figure 5 shows a 'closed loop' concept of the FA approach in a WWTP. A portion of the activated sludge from the mainstream bioreactor or from the sludge return line is transferred to an FA treatment unit on a daily basis. In the FA treatment unit, the NOB completely lose their activity but the AOB activity is only partially affected. Afterwards, the FA-treated sludge is

returned to the mainstream bioreactor, in which the NOB would be washed out gradually (in around 1 month) and the improved nitrogen removal performance would be achieved. The FA required for the proposed approach is a renewable chemical and is directly attainable from the anaerobic digester effluent (a by-product of wastewater treatment) of WWTPs. Anaerobic digester effluent has an ammonium level of 1.0~2.0 g N/L and is at a pH of 7.5~8.6 and 33 °C.[17,18] This is equivalent to an FA level of 30~560 mg NH_3-N/L. This would be adequate for the proposed FA approach (i.e. 210 mg NH_3-N/L). Therefore, no additional nitrogen load would be introduced to the WWTPs. In cases that the desirable FA level is not achieved in the FA treatment unit, moderate amount of alkali can be added to further raise the pH and hence the FA level. After being returned to the mainstream bioreactor, the FA can be significantly diluted by 3~4 orders of magnitude and removed by nitrification and denitrification. Therefore, this FA approach would not have any adverse impact on the performance of the mainstream bioreactor. The FA approach also sets an excellent example for the paradigm shift of the WWTPs from 'linear economy' to 'circular economy' because FA come from the anaerobic digester effluent, which is a waste of WWTPs.

(Position for Figure 5)

Implications of Proposed FA Approach for Wastewater Treatment

The proposed FA approach will be highly beneficial for the WWTP deficient in COD for nitrogen removal. It would potentially reduce oxygen demand, decrease COD requirement and also reduce sludge production. In order to evaluate the economic feasibility of the proposed FA approach, the experimental results acquired in this study were used to conduct the economic

363 analysis of the FA approach. This was conducted in the full-scale WWTP without sufficient
364 COD for nitrogen removal via nitrate using a desktop scaling-up analysis (see Table S1 in
365 Supporting Information). The economic analysis results were described in Table S1. Compared
366 to the WWTP without the FA approach, the WWTP with the FA approach would save
367 $2.6/PE/year (Table S1; PE: population equivalent). The saving was because of the decreased
368 aeration energy cost (i.e. saving $0.4/PE/year), saved cost for methanol addition (i.e. saving
369 $2.0/PE/year) and reduced cost of sludge transport and disposal (i.e. saving $0.4/PE/year)
370 deducting the extra cost (i.e. $0.2/PE/year) for sludge treatment using FA. Therefore, the FA
371 approach is economically beneficial. The benefit of the potential sludge reduction in the
372 wastewater treatment line is not included in this economic analysis because the sludge reduction
373 in the wastewater treatment line cannot be quantified for the time being (see the fifth paragraph
374 of this Section). Therefore, the potential economic benefit of the FA approach would be even
375 larger. Nevertheless, it should be noted that the results of economic evaluation reported in this
376 work should be regarded indicative only due to the fact that the actual cost might be variable
377 relying on local conditions.
378
379 For the WWTP with sufficient COD for nitrogen removal, the proposed FA approach will also
380 be beneficial. From the integrated economic and environmental point of view, the carbon source
381 in a WWTP should be manipulated in order to attain not only desirable nitrogen removal
382 performance but also large energy recovery via anaerobic methane production. When the
383 nitrogen removal through nitrite pathway was attained via the FA approach, a carbon source
384 saving of 40% for biological nitrogen removal would be achieved theoretically.[1] With this saving,
385 the primary settler can be reinstalled in the WWTPs which previously abolished primary settler

386 for improving nitrogen removal. The coagulant can also be added to the wastewater. These will
387 enhance the separation of particulate and colloidal organics from wastewater for methane
388 generation through anaerobic digestion without affecting nitrogen removal performance.[23]
389 Therefore, the 'carbon source challenge' problem confronted by the WWTPs could be
390 potentially solved by the FA approach.
391
392 The potential environmental impact of the proposed FA approach was also analyzed in terms of
393 CO_2 emission. In comparison to the WWTP without the FA approach, the overall CO_2 emission
394 was estimated to decline by 12.3 kg CO_2/PE/year (see Table S1 in Supporting Information). This
395 could be attributed to the decreased aeration energy consumption (3.0 kg CO_2/PE/year), avoided
396 industrial methanol production (2.6 kg CO_2/PE/year) and methanol oxidization (6.7 kg
397 CO_2/PE/year) after implementing FA approach despite the alkali consumption (leading to
398 negligible CO_2 emission i.e. 7e-5 kg CO_2/PE/year). Consequently, the FA approach could be
399 considered environmentally friendly. However, it should be noted that N_2O emission was not
400 included in the environmental analysis.
401
402 The effective NOB elimination using FA also potentially provides a solution to the achievement
403 of stable mainstream autotrophic nitrogen removal by partial nitritation followed by anaerobic
404 ammonium oxidation (Anammox, $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$).[24,25] This nitrogen removal process
405 does not require carbon source, thereby being able to achieving the upfront separation of carbon
406 source for maximal methane production while achieving satisfactory nitrogen removal. However,
407 the major barrier for the above autotrophic nitrogen removal process is the suppression of
408 NOB.[24,26] Therefore, the proposed FA approach might also be effective in eliminating NOB in

409 the mainstream autotrophic nitrogen removal system, which will require further study in the
410 future. When FA approach is implemented in the mainstream autotrophic nitrogen removal
411 system, it is expected that Anammox will not be affected. This is because only the sludge from
412 the partial nitritation reactor (i.e. without Anammox bacteria) would be treated in the FA
413 treatment unit in the two-stage partial nitritation-Anammox mainstream autotrophic nitrogen
414 removal system. In the one-stage system, FA approach would not affect Anammox either. This is
415 because Anammox bacteria grow in granules or on carriers in a one-stage system, which could
416 be easily separated from floccular sludge where AOB and NOB grow.
417
418 This study also demonstrates that the sludge concentration in the SBR was reduced by 18% after
419 implementing FA treatment. This indicates that sludge treatment using FA might enhance the
420 sludge biodegradability. Indeed, Wei et al. [19] found that anaerobic methane production from
421 waste activated sludge was increased after the waste activated sludge was pretreated by FA at
422 250 mg NH_3-N/L for 1 day because of the enhanced sludge biodegradability. This reveals that
423 FA treatment might also be used as a strategy for reducing sludge production in the wastewater
424 treatment line of the WWTPs. This can be achieved by implementing FA treatment on return
425 activated sludge. Also, a longer SRT should be applied to the system with FA treatment but
426 without the corresponding increase in the MLSS concentration due to the enhanced sludge
427 biodegradability. Then, the sludge production from the two systems with and without FA
428 treatment can be objectively compared.[27] The reduction in sludge production can then be
429 quantified.
430

This study shows that around 10.5 mg NO_2^--N/L was observed in the effluent after eliminating NOB and establishing the nitrogen removal through nitrite pathway (Figure 4 and Table 1). It has been reported that nitrite could impose harmful impact on the receiving water since it is toxic to the aquatic animals. To address this issue, the step-feed strategy could be used. The step-feed strategy has been demonstrated to be effective in reducing the toxic nitrite level in the effluent while the nitrogen removal was via the nitrite pathway. [13] Also, pre-denitrification (i.e. anoxic reaction is followed by aerobic reaction) was adopted in this study, which is also the most commonly used process in WWTPs. This contributed to the observed effluent nitrite and nitrate although the carbon source is not limiting after implementing FA treatment.

We would like to point out that the FA approach demonstrated in this work differed from previously the reported FA method (e.g., Vlaeminck et al [28]). The work of Vlaeminck et al. [28] depended on FA inhibition on NOB, where above 3.0 mg NH_3-N/L (equivalent to 800 mg NH_4^+-N/L at pH 7.0 and 20 ˚C) was applied. However, the study of Vlaeminck et al. [28] requested constant existence of FA at > 3.0 mg NH_3-N/L in the bioreactor. This would be impossible to achieve in the mainstream bioreactor receiving domestic wastewater with a low nitrogen load (< 60 mg NH_4^+-N/L). In contrast, our work was dependent on NOB inactivation, which only required FA in a small separate sludge treatment unit instead of in the mainstream bioreactor. Therefore, it was easy to achieve, as demonstrated in this work.

Wang et al. [13] previously demonstrated that sludge treatment using FNA was effective in washing out NOB and establishing the nitrogen removal through nitrite pathway. However, the FNA approach relied on the FNA production on site (avoiding external nitrogen input) through

side stream nitritation of anaerobic digester effluent.[13] Unfortunately, most of the WWTPs does not have the side stream nitritation reactor and therefore FNA production on site would be difficult. In contrast, the proposed FA approach in this work does not rely on the side stream nitritation reactor. The essential chemical (that is FA) for this approach can be directly obtained from the anaerobic digester effluent. As a result, the FA approach is more suitable for the WWTPs lack of the side stream nitritation reactor, whereas the FNA approach is more suitable for the WWTPs with the side stream nitritation reactor. In addition, a nitrite accumulation ratio of ~80% was achieved using the FNA approach,[13] whereas the FA approach achieved a higher nitrite accumulation ratio (i.e. ~100%). It should be noted that although the FA approach was investigated using an SBR in this work (because of the equipment availability), it was not dependent on any SBR features and was also applicable to a wide range of WWTPs.

In this study, technology optimization was not conducted. For example, only one FA concentration (i.e. 210 mg NH_3-N/L), one treatment frequency (i.e. 0.29) and one treatment time (i.e. 1 day) were tested. This is because the aim of this study is to demonstrate the feasibility of achieving nitrogen removal via the nitrite pathway by sludge treatment using FA. Also, a number of parameters (e.g. FA concentration, treatment time and sludge treatment frequency) can be optimized. Therefore, the scope of this optimization would be very large and would require a comprehensive study that cannot be accommodated in this initial proof-of-concept study. In the future, technology optimization will be conducted. Intermittent FA treatment strategy would also be developed in the following study. In addition, Table 1 and Figures. 2 and 4 reveals that AOB activity was not negatively affected and a minor fraction of NOB still existed in the SBR although the batch test results (Section: Inactivation of NOB and AOB by FA) indicated an AOB

activity loss of 50% and a complete NOB activity loss following FA treatment. This was probably attributed to the fact that the average sludge treatment frequency was 0.29 per reactor volume per day (see Section "Reactor Operation and Monitoring"). This would enable both AOB and NOB to have an average recovery/regrowth time of 3.4 d (i.e. 1/0.29) in the SBR following FA treatment. The complete washout of NOB and an even higher nitrite accumulation ratio (i.e. ~100%) would be potentially achievable after technology optimization. Also, if this FA approach is implemented in an enhanced biological phosphorus removal plant, it might affect phosphorus removal and this requires further investigation. It should also be pointed out that while excellent results were attained in our laboratory studies, full-scale tests are still required to fully reveal the feasibility and potential of this approach.

In summary, the effectiveness of sludge treatment using free ammonia (FA) in washing out nitrite oxidizing bacteria and attaining nitrogen removal via the nitrite pathway was investigated via a long-term laboratory test. The NOB is inactivated by FA to a much larger extent compared with AOB; so that it can be effectively eliminated and thus nitrogen removal via the nitrite pathway could be efficiently established in the mainstream bioreactor. This could be achieved by treating a portion of the activated sludge using FA, together with the subsequent recirculation of the FA treated sludge to the mainstream activated sludge system. Nitrogen removal performance could be significantly improved in the mainstream bioreactor by the established nitrite pathway using the FA approach. The FA approach for eliminating the nitrite oxidizing bacteria and achieving the nitrogen removal through nitrite pathway is economically and environmentally favorable.

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Notes

The authors declare no competing financial interest.

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ASSOCIATED CONTENT

Supporting Information Available

One table summarizing the detailed economic and environmental analysis results are shown in Supporting Information. This information is available free of charge via the Internet at http://pubs.acs.org/.

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600 **List of Figures and tables**

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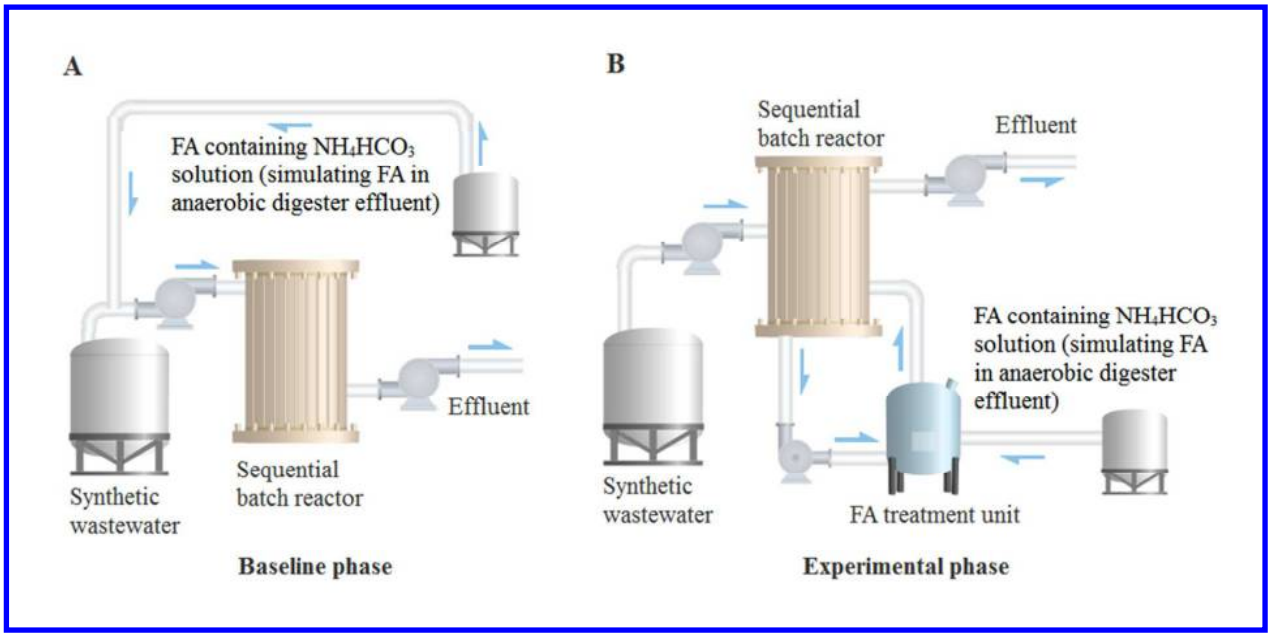

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603 **Figure 1.** Schematic diagram of the experimental setup during the Baseline phase (A) and

604 Experimental phase (B).

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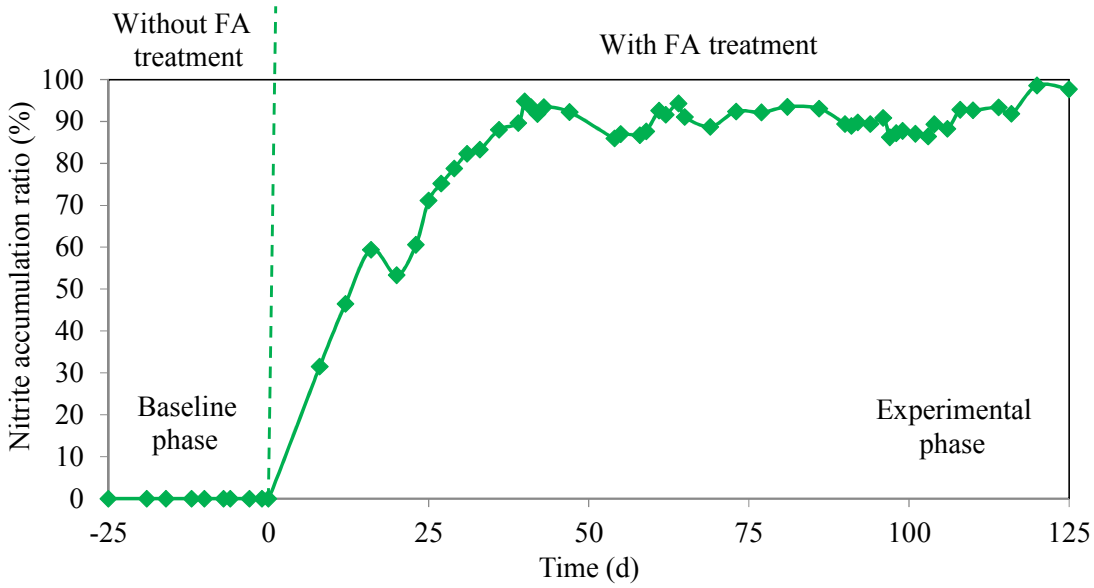


Figure 2. Nitrite accumulation ratio (NO_2^--N/(NO_2^--N+NO_3^--N)×100%) in the effluent of the reactor before and after implementing FA treatment.

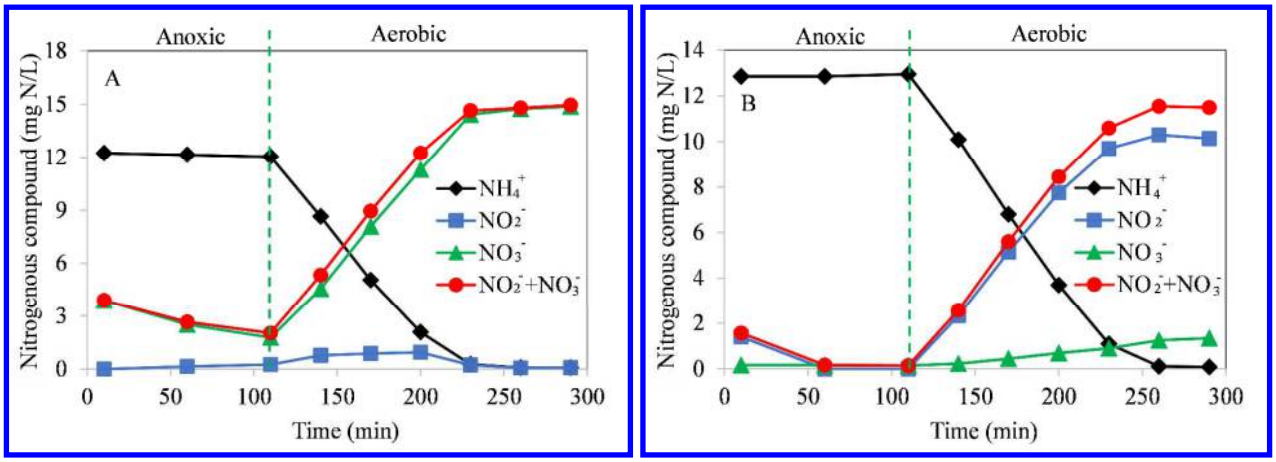
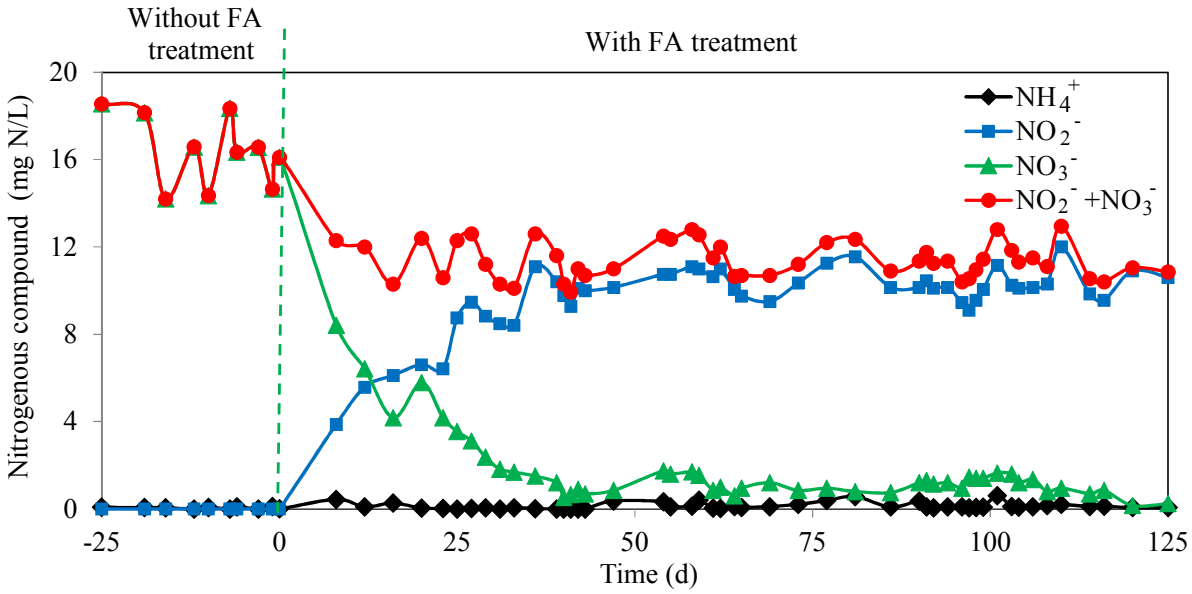


Figure 3. Variations in the concentrations of NH_4^+-N, NO_2^--N, NO_3^--N and NO_2^--N + NO_3^--N during a typical cycle in the reactor before (A) and after (B) implementing FA treatment.



639 **Figure 4.** Long-term NH_4^+-N, NO_2^--N, NO_3^--N and NO_2^--N + NO_3^--N concentrations in the

640 effluent of the reactor.

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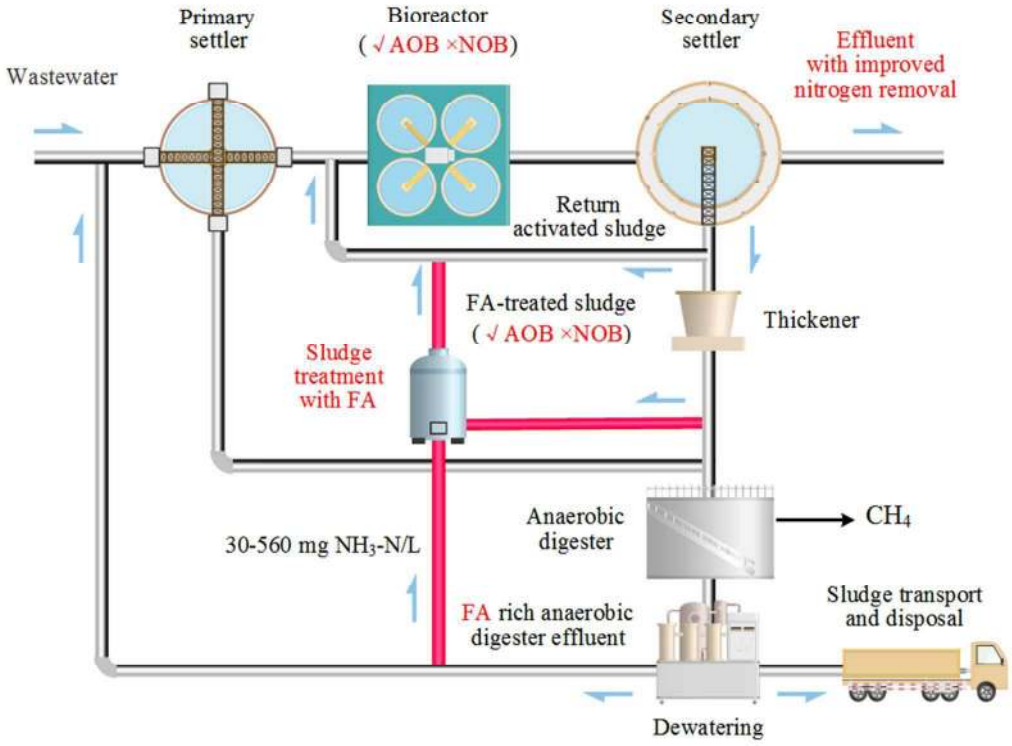

Figure 5. The innovative 'closed loop' concept in a WWTP based on the proposed FA approach to selectively wash out nitrite oxidizing bacteria and achieve the nitrite pathway.

658 **Table 1.** Reactor performance and sludge characteristics of the SBR at Steady States I (without
659 FA treatment) and II (with FA treatment) (with standard errors).

Parameter		Steady State I	Steady State II
Reactor performance	Effluent NH_4^+ concentration (mg N/L)	0.1 ± 0.1	0.1 ± 0.1
	Effluent NO_2^- concentration (mg N/L)	0.0 ± 0.0	10.5 ± 0.1
	Effluent NO_3^- concentration (mg N/L)	16.4 ± 0.5	1.0 ± 0.1
	Effluent NO_x^- concentration (mg N/L)[a]	16.4 ± 0.5	11.5 ± 0.1
	NO_2^--N accumulation ratio (%)[b]	0.0 ± 0.0	91.3 ± 0.5
Sludge characteristics	Activity of AOB (mg NH_4^+-N/g MLVSS/h)	9.6 ± 0.6	8.7 ± 0.7
	Activity of NOB (mg NO_3^--N/g MLVSS/h)	8.1 ± 0.7	0.2 ± 0.1
	AOB population (%)	6.9 ± 1.6	7.6 ± 1.3
	NOB population (%)	2.7 ± 0.5	0.2 ± 0.1
	MLSS (mg/L)	1048 ± 57	850 ± 54
	MLVSS (mg/L)	998 ± 51	795 ± 39
	SVI (ml/g)	193 ± 25	244 ± 24

660 [a] NO_x^--N= NO_2^--N+NO_3^--N

661 [b] NO_2^--N accumulation ratio=NO_2^--N/NO_x^--N×100%